Polyacrylamide gel electrophoresis: a powerful tool in the food-processing sector

Nancy Álvarez Mittelmann[1], Eva Diago Saavedra[1], Eva Mª García Calvo[1], Azahara Montero Méndez[1], Juan Carlos Pardo Carmona[1], Sandra Pérez López[1], Estefanía Soriano Calvo[1], Cristina Tortosa Jiménez[1], Carmelo Ruiz[2], Francisco Javier Corpas[2], José Manuel Palma[2]

[1]*IES Zaidín Vergeles, Avda. Primavera s/n. Granada*
[2]*Group of Antioxidants, Free Radicals and Nitric Oxide in Biotechnology and Agricultural Food. Department of Biochemistry, Cell and Molecular Biology of Plants, Estación Experimental del Zaidín, CSIC, Granada.*
e-mail: carmelo.ruiz@eez.csic.es

SUMMARY

Polyacrylamide gel electrophoresis (PAGE) is an analytical and powerful technique widely used in research to analyze proteins and nucleic acids. In this project, this technique has been used to study the protein pattern in different organs (leaves and fruits) and plant species. Specifically, we used pepper and pea plants, which have agronomical interest, and Arabidopsis as a model plant. Two different applications of PAGE, designated as SDS-PAGE and non-denaturing PAGE, useful to investigate the pattern of polypeptides and to assay in situ the enzymatic activities, particularly superoxide dismutase (SOD), were developed, respectively. The results show that each plant sample has a specify protein pattern and the number and type of SOD isoenzymes varies depending on the organ and the plant species.

INTRODUCTION

Polyacrylamide gel electrophoresis (PAGE) is an analytical and powerful technique widely used in research for proteins and nucleic acids. Nowadays, there are two main types of gel electrophoresis: one dimension and two dimensions. One dimension PAGE includes **SDS-PAGE** which is the most widely used electrophoresis technique to separate proteins primarily by mass. In this type of electrophoresis the samples are heated, so proteins are denatured by breaking of the disulfide bonds and simultaneous treatment with SDS (sodium dodecyl sulfate), an anionic detergent, gives negative charge to each protein in proportion to its mass. Thus, when a current is applied, all SDS-bound proteins in a sample will migrate through the gel towards the positively charged electrode. Proteins with less mass migrate more quickly through the gel than those with greater mass because of the sieving effect of the gel matrix. On the other hand, non denaturing PAGE, also called **native PAGE**, separates proteins according to their mass/ charge ratio. This technique allows detecting *in situ* some enzymatic activities. In the case of two-dimensional PAGE (2D-PAGE), proteins are separate by sequentially by their isoelectric point in the first dimension, also called isoelectric focusing (IEF), and by the mass in the second dimension, as in the SDS-PAGE.

In this project, several of these techniques have been used to study the protein pattern in plant samples from different origins. In particular, we have used pepper and pea plants which have agronomical interest and *Arabidopsis thaliana* as a model plant. Thus, we have two different applications of PAGE designated as SDS-PAGE and non-denaturing PAGE which are useful to distinguish the pattern of polypeptide and to assay *in situ* an enzymatic activity, specifically superoxide dismutase (SOD).

Superoxide radical ($O_2^{\cdot -}$) is generated as a by-product in aerobic organisms from a number of physiological reactions such as the electron flow in the chloroplasts and mitochondria and from some redox reactions in cells. It can react with hydrogen peroxide (H_2O_2) to produce hydroxyl radical ($\cdot OH^-$), one of the most reactive molecules in the living cells. Hydroxyl radical can cause the peroxidation of membrane lipids, breakage of DNA strands, and inactivation of enzymes in cells (for reviews, see Bowler

et al., 1992; Mehdy, 1994). To ameliorate the damage caused by hydroxyl radical formed from superoxide radical and hydrogen peroxide, organisms have evolved mechanisms to control the concentration of the two reactants. Superoxide dismutase (SOD) is a group of isozymes functioning as a superoxide radical scavenger in the living organisms. The reaction catalyzed by SOD is as follows:

$$O_2^{-} + O_2^{-} + 2H^{+} \xrightarrow{\text{SOD}} H_2O_2 + O_2$$

There are three types of SOD isozymes which are classified according to the metal at the catalytic center, copper and zinc (CuZn-SODs), manganese (Mn-SODs) or iron (Fe-SODs). The most abundant SODs in plants are the CuZn-SODs, which are found mainly in the cytosol and chloroplasts. SOD is an important enzyme family in living cells for maintaining normal physiological conditions and under several stress conditions.

SPECIFIC AIMS

1. Protein extraction and separation from different organs and plant species using denaturing and non-denaturing polyacrylamide gel electrophoresis.
2. After SDS-PAGE and protein staining, analysis of protein pattern in plant samples
3. After non-denaturing PAGE, analysis of superoxide dismutase (SOD) and identification of number and type of the different SOD isozymes present in the plant samples.

MATERIALS AND METHODS

Plant materials and growth conditions

Pepper (*Capsicum annuum* L., type Dulce italiano) and pea (*Pisum sativum* cv Lincoln) plants of approximately 30-days were grown in aerated optimum nutrient solution (Airaki et al. 2011). *Arabidopsis thaliana* ecotype Columbia seeds were germinated and grown on soil plus vermiculite (1:3). Plants were grown in a growth chamber at 24 °C, under a 16 h photoperiod and a light intensity of 190 µE m^{-2} s^{-1} for a period of 4 weeks. Pepper fruits were purchased at the local market (Fig. 1).

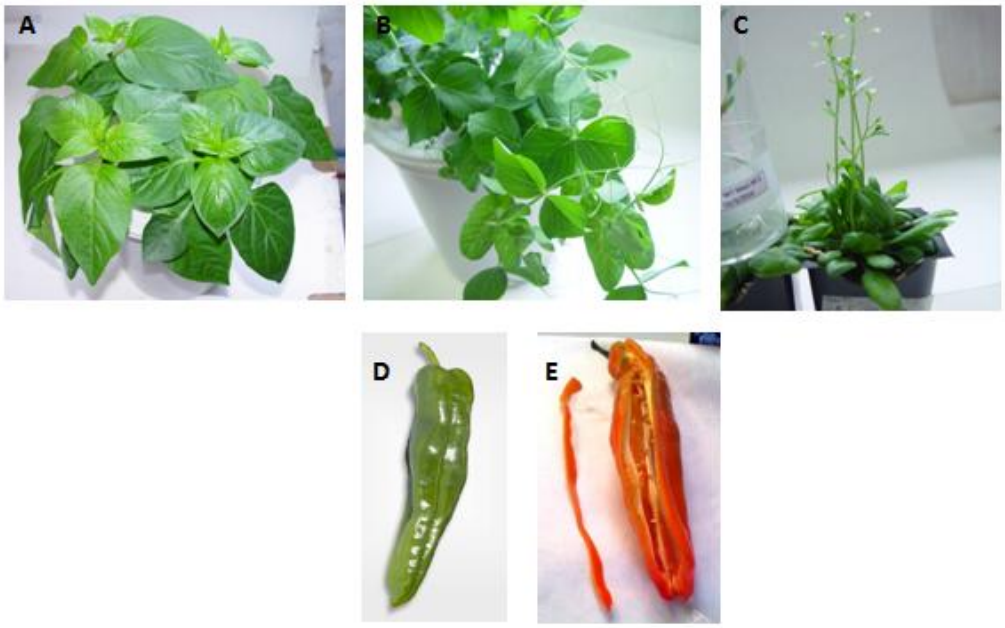

Figure 1. Phenotype and appearance of the plant materials used in our experiments. (A) Pepper plants. (B) Pea plants. (C) *Arabidopsis* thaliana plants. (D and E) Pepper fruits.

Crude extracts of plant tissues

The plant samples were homogenized (relation 1/3; w/v) in assay tubes in buffer 50 mM Tri-HCl, pH 7.8 containing 10% (v/v) glycerol, 0.1 mM EDTA and 0.2 % (v/v) Triton X-100 (Table 1). Then, samples were filtered through two nylon layers and centrifuged at 27,000 g for 26 min at 4 °C. The obtained supernatants were used for the assays.

Table 1. Plant material used

Sample	Fresh weight (g)	Buffer (mL)
Green pepper	0.68	2.04
Red pepper	1.78	5.34
Pepper leaves	1.06	3.18
Pea leaves	0.98	2.94
Arabidopsis leaves	0.49	1.47

Protein concentration

Protein content was determined according to the method of Bradford (1976) using the commercial Bio-Rad Protein Assay (Hercules, CA) kit, with bovine serum albumin (BSA) as standard. In this method the Bradford dye (Coomassie Brilliant Blue G-250) forms a complex with the proteins and the increase of absorbance at 595 nm is proportional to the amount of bound dye (Bradford, 1976).

The calibration curve was prepared with using 0, 3, 6, 12 and 18 µg BSA in a final volumes of 800 µL. Then, 200 µL of the BioRad reagent were added given a final volume of 1 mL (Fig. 2). The plant samples (pea, pepper and Arabidopsis) were prepared in similar volume with adequate dilutions. After 10 to 15 min, the absorbance at 595 nm was measured in a spectrophotometer Beckman. The concentration was determined usinf the calibration curve (Fig. 2).

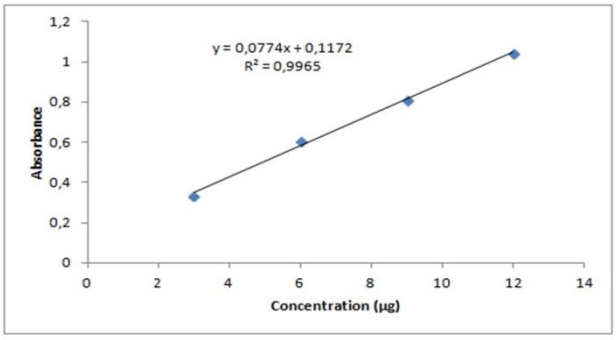

Figure 2. Calibration curve obtained by method of Bradford using BSA as standard

SDS-PAGE and protein staining with Coomassie Blue

Four polyacrylamide gels were prepared in a Mini – Protean III equipment of Bio Rad (Fig. 3), according to the indications given in Table 2. The electrophoresis samples were prepared in a loading buffer made with 125 mM Tris – HCl, pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 0.006 % (w/v) bromophenol blue and 10 mM DTT, and the protocol indicated in Table 3 was followed.

Before loading, samples were heated in boiling water for 5 min.

After that, the gels were run at 100 V for 15 – 20 min; then, we applied 200 V for 45 min. The electrode buffer used for the cathode was 2.5 mM Tris – HCl, pH 8.3, 0.192 M glycine and 0.1% (w/v) SDS. The anode buffer was similar but without SDS. The amount of sample loaded onto gels depended of the sensibility of the staining method that we used.

Gels were stained with 0.1% (w/v) "Coomassie Brillant Blue" R-250,. This was prepared in 50% (v/v) methanol and 10% acetic acid . The staining of gels was run for for 30 min. Then, gels were clarified with 40% (v/v) methanol and 10% (v/v) acetic acid, until blue band appeared over a colourless background.

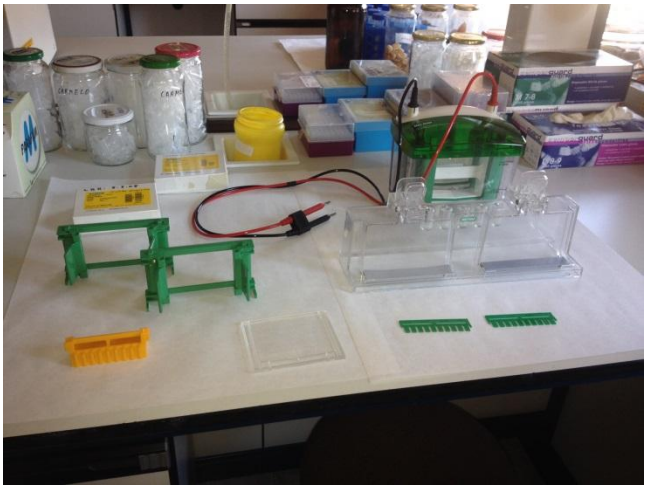

Figure 3. Components of a vertical electrophoresis cell (BioRad) used in our experiments

Table 2. Gel composition for running and stacking gels in SDS_PAGE.

	Running gel (12 %)	Stacking gel (4 %)
30 % Acrylamide	3.7 mL	0.50 mL
4 × Running gel	2.3 mL	-
4 × Stacking gel	-	1.00 mL
10 % (w/v) SDS	90 µL	40 µL
50 % (v/v) Glycerol	0.9 mL	-
Milli-Q H_2O	2.1 mL	2.46 mL
VACUUM 10 min		
10 % (w/v) Ammonium persulfate	50 µL	50 µL
TEMED	5 µL	10 µL

Table 3. Samples distribution in for loading SDS-PAGE gels. SDS, sodium dodecyl sulfate. M, Molecular Weight Markers.

1st well	2nd well	3rd well	4th well	5th well	6th well	7th well	8th well	9th well	10th well
-	M	SDS	Pea leaves	Pepper leaves	Green pepper fruits	Red pepper fruits	Arabidopsis leaves	SDS	-
-			30 µL	30 µL	30 µL	30 µL	30 µL	30 µL	-

Non-denaturing PAGE and superoxide dismutase activity

In this electrophoresis, proteins were exposed to migration under native conditions. Proteins migrate depending on their charge, size and form.

We used a BIO Rad Mini-Protean III equipment where gels of 6.5 x 8 cm and 1.5 mm of thickness were made. Gels were prepared with a 10 % (w/v) polyacrylamide concentration of in Tris-HCl 377 mM, pH 8.9 buffer, as indicated in Table 4. Sample homogenates were prepared in 10 % (v/v) glycerol and 0.006 % (w/v) bromofenol blue and loaded directly onto gels as shown in Table 5, . We used an intensity of 15 mA for gel during 30 min, and then 25 mA until the blue of bromofenol reached practically the end of the gel. The electrode buffer used was 38 mM glycine, adjusted to pH 8.2 with the gel buffer.

SOD isoenzymes were identified in gels by a photochemical method. Gels were stained with 2.45 mM NBT for 20 minutes with constant agitation, followed by an 15 minutes incubation in a solution of µM 28 riboflavine, 28 mM TEMED in 50 mM potassium phosphate buffer (pH 7.8). The incubation were performed in darkness. Developing of gels was carried out with visible light during 15 min at room temperature.

Table 4. Gel composition for non-denaturing PAGE.

Gel 10 %	
30 % Acrylamide	9.00 mL
Gel buffer (1.5 M)	6.75 mL
Milli-Q H_2O	9.85 mL
VACUUM 10 min	
7 % (w/v) Ammonium persulfate	1.45 mL
TEMED	100 µL

Table 5 Samples distribituion in non-denaturing PAGE

A.

1st well	2nd well	3rd well	4th well	5th well	6th well	7th well	8th well	9th well	10th well
-	-	Pea leaves	Arabidopsis leaves	-	Pepper leaves	Green pepper fruits	Red pepper fruits	-	-
-	-	40 µL	50 µL	-	50 µL	50 µL	50 µL	-	-

RESULTS

SDS-PAGE and protein staining

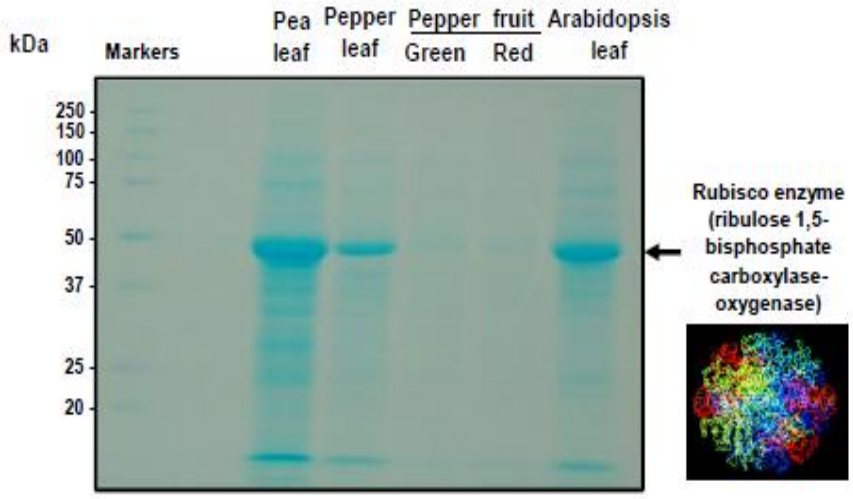

Figure 4. Electrophoretic polypeptide patterns under denaturing conditions using different types of plant samples. The migration of the most abundant protein in leaves (Rubisco) is indicated.

Non-denaturing PAGE and superoxide dismutase (SOD) activity

Figure 5. Electrophoretic pattern of SOD isozymes from distinct plant samples.

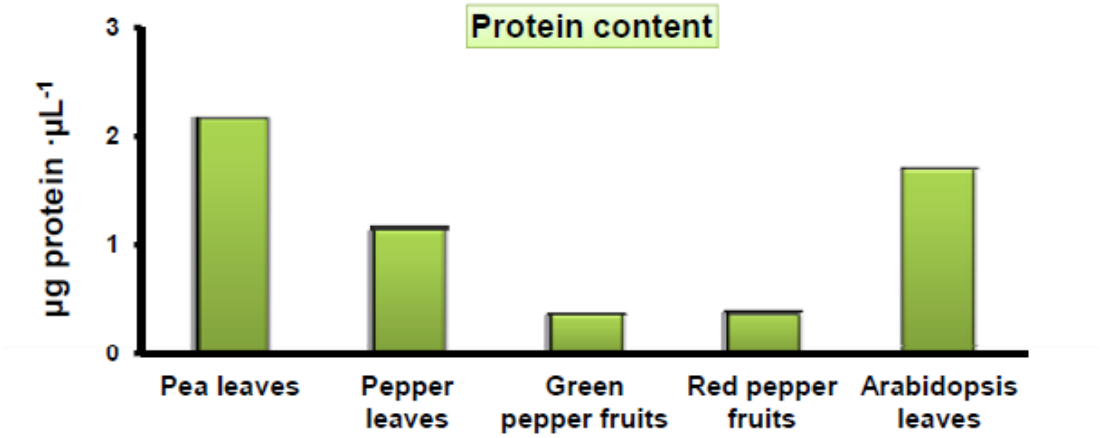

Figure 6. Protein concentration (expressed as µg/ µL) of the five plant samples studied in this work.

CONCLUSIONS

1. The protein content in fruits is lower than in leaves and this could be due to the higher water content in pepper fruits.
2. The protein pattern is different depending on the plant species and the analyzed organs. The most prominent band in leaves is the Rubisco which is present in chloroplasts and participates in the photosynthesis.
3. We have identified until four superoxide dismutase (SOD) isoforms in pepper fruits but this pattern changes depending of the analyzed organs and the plant species.

ACKNOWLEDGEMENTS

This work was supported by the ERDF-cofinanced grant AGL2011-26044 from the Ministry of Science and Innovation, Spain. Antonio Quesada Ramos, Professor at the IES Zaidín-Vergeles, Granada is specially acknowledged for his continuous assistance to develop our work.

REFERENCES

[1] Airaki M, Leterrier M, Mateos RM, Valderrama R, Chaki M, Barroso JB, Del Río LA, Palma JM, Corpas FJ (2012) Metabolism of reactive oxygen species and reactive nitrogen species in pepper (*Capsicum annuum* L.) plants under low temperature stress. *Plant Cell and Environment* 35: 281-295.
[2] Asada K (2006) Production and scavenging of reactive oxygen species in chloroplasts and their functions. *Plant Physiology* 141: 391–396.
[3] Bambeck SG (1996) Electrophoresis separation gel and method for preparing an electrophoresis separation gel. USA patent number. 5 589 104.
[4] Bradford MM (1976). Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72: 248–254.
[5] Coligan JE, et al. , Eds. (2002) Electrophoresis, In Current Protocols in Protein Science, pp. 10.0.1-10.4.36. John Wiley and Sons, Inc. New York.
[6] Hames BD, Rickwood D, Eds. (1990) Gel Electrophoresis of Proteins: a Practical Approach, 2nd ed. Oxford University Press, New York.

[7] Beauchamp C, Fridovich I (1971) Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Analytical Biochemistry* 44: 276–287.

MY OWN IDEAS

Nancy Álvarez Mittelmann, IES Zaídin-Vergeles

A mí personalmente, me parece fantástica toda esta tecnología desarrollada y aplicada a moléculas orgánicas para poder analizarlas. Con el desarrollo de la tecnología cada vez nos vamos adentrando en el mundo de lo más pequeño para poder entender y saber cómo funcionamos.

Ésta fue una experiencia positiva para mí ya que fue mi primer contacto del mundo de la investigación. Tuve el privilegio de trabajar junto a investigadores y corroborar que la investigación es primordial para el desarrollo de un país. El futuro es la biotecnología y dentro de las técnicas que engloba, la electroforesis es una de ellas.

En este proyecto, como el título indica, la electroforesis es de gran utilidad en el sector agrícola-alimentario, dado que podemos aplicar este proceso para identificar proteínas. El DNA contiene la información necesaria para sintetizar proteínas, por tanto, con la identificación de las proteínas, podemos diferenciar a qué gen pertenece. Por supuesto, este proceso se complica dado que un gen puede dar lugar a varias proteínas.

Centrándonos de nuevo en la idea que estamos tratando en este caso con hortalizas y vegetales, si por ejemplo, podemos identificar una proteína que desarrolle una característica de resistencia a la sequía (un mal muy frecuente dada nuestra climatología española), podríamos ser capaces de incorporar esta resistencia a otras plantas, que a su vez daría lugar a un ahorro enorme en el agua en el sector de la agricultura. Esta técnica, de poder realizarse, también se podría extrapolar a casos en donde las hortalizas a tratar sean vulnerables a plagas y a su vez, un menos uso de fertilizantes que provocan impactos medioambientales en el suelo.

Eva María García Calvo, IES Zaidín-Vergeles

Si uno de los objetivos del proyecto PIIISA es acercar el estudio de las ciencias al alumnado e incentivarles en la investigación, lo consiguen con creces.

Este proyecto me ha abierto la mente a un nuevo campo de estudio que en un principio no me llamaba mucho la atención. Las plantas, su proceso de maduración, la elección de muestras, el tratamiento cuidadoso y escrupuloso con ellas, poder "ver" las proteínas que tienen y cuantificarlas, y además aprender a usar las herramientas adecuadas para descubrir hechos desconocidos. Es apasionante el mundo de la investigación, ahora lo sé.

Siendo alumna de un ciclo de FP, agradezco el esfuerzo por parte de la Estación Experimental del Zaidín del CSIC, del departamento de bioquímica, biología celular y molecular de plantas y de los investigadores Carmelo Ruiz, Francisco Javier Corpas y José Manuel Palma por hacer posible que esta experiencia haya llegado a nosotros. Si hubiera sido posible, habría participado en él mucho antes y espero que este proyecto dure muchos años y siga acercando la ciencia a nuestros futuros y pequeños Einsteins.

"Escucha, mira, huele, toca, gusta… el mundo está ahí para descubrirlo."

Azahara Montero Méndez, IES Zaidín Vergeles

Este proyecto nos ha dado la oportunidad de realizar una técnica de electroforesis en geles de poliacrilamina y aplicarla al análisis bioquímico y molecular de productos hortofruticulas permitiendo la obtención de un panel de marcadores proteicos de calidad.

Me siento orgullosa de haber participado en este proyecto, ya que nos han dado la oportunidad de adentranos de lleno en el mundo de la investigación, así como de usar sus instalaciones, material y medios especializados.

Creo que el proyecto PIIISA es una gran oportunidad de mostrar a los jóvenes el mundo de la ciencia y la investigación.

Por último, destacar que nos hemos sentido muy cómodos con los investigadores del CSIC, y agradecerles su esfuerzo y el tiempo que nos han dedicado para hacer posible este proyecto.